. ATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

INVITATION TO PAY ADDITIONAL FEES

(PCT Article 17(3)(a) and Rule 40.1)

To:
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REGISTERED

Date of mailing (day/month/year): 02/06/2000

Applicant's or agent's file reference: 341.012WO1

PAYMENT DUE within 45 ~~XXXXX~~s/days from the above date of mailing

International application No.: PCT/US 99/ 30925

International filing date (day/month/year): 22/12/1999

Applicant: PROMEGA CORPORATION

July 17, 2000

1. This International Searching Authority

(i) considers that there are 3 (number of) inventions claimed in the international application covered by the claims indicated ~~XXXX~~ on the extra sheet:

and it considers that the international application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated ~~XXXX~~ on the extra sheet:

(ii) [X] has carried out a partial international search (see Annex) [ ] will establish the international search report on those parts of the international application which relate to the invention first mentioned in claims Nos.:

See additional Sheet, Invention 1.

(iii) will establish the international search report on the other parts of the international application only if, and to the extent to which, additional fees are paid

2. The applicant is hereby **invited,** within the time limit indicated above, to pay the amount indicated below:

EUR 945.00 (Fee per additional invention) x 2 (number of additional inventions) = EUR 1,890.00 (total amount of additional fees)

Or, _____ x _____ = _____

The applicant is informed that, according to Rule 40.2(c), **the payment of any additional fee may be made under protest,** i.e., a reasoned statement to the effect that the international application complies with the requirement of unity of invention or that the amount of the required additional fee is excessive.

Schwegman, Lundberg, Woessner & Kluth, P.A.

JUN 07 2000

3. [X] Claim(s) Nos. See Remark have been found to be unsearchable because Article 17(2)(b) because of defects under Article 17(2)(a) and therefore have not been included with any invention.

RECEIVED

Name and mailing address of the International Searching Authority

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Authorized officer

Andria Overbeeke-Siepkes

Form PCT/ISA/206 (July 1992)

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (1-10,12-46,65-68,73)-complete, (11,58)-partially

A second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase; said second luciferase which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase; said second luciferase wherein the reference luciferase is LucpPe-2 (luciferase from Photuris pennsylvanica); luciferases: luc 133-1B2 and luc146-1H2 which comprise SEQ ID Nos. 44 and 45; an isolated and purified nucleic acid molecule comprising a nucleic acid sequence selected from SEQ ID Nos.42 and 43; a vector comprising said nucleic acid molecule; a host cell comprising said vector; a fusion protein comprising said luciferase; the use of the second luciferase for detecting ATP, for labeling a molecule, as a genetic reporter, for immobilization onto a solid surface, to produce a hybrid molecule, for high temperature reactions, or for creating luminescent solutions; a method for using said vector encoding said luciferase; a kit comprising: a container comprising said second luciferase; method to prepare an luciferase that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding luciferase which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding luciferase subjected to conditions that yield nucleotide mutations, wherein the luciferase encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the luciferase encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding luciferase that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the first polynucleotide sequence encodes Ppe2 and/or Ppl;

1.1. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc49-7C6, respectively SEQ ID Nos. 1 and 14, where the reference beetle luciferase is lucPpe-2;

1.2. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc78-0B10, respectively SEQ ID Nos. 6 and 19, where the reference beetle luciferase is lucPpe-2;

1.3. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc90-1B5 respectively SEQ ID Nos. 11 and 24, where the reference beetle luciferase is lucPpe-2;

1.4. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-6G01 respectively SEQ ID Nos. 13 and 26, where the reference beetle luciferase is lucPpl;

1.5. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to luc81-0B11, respectively SEQ ID Nos. 46 and 47, where the reference beetle luciferase is lucPpl;

1.6. Claims: (1-10,12-46,65-68,73)-complete, (11, 58)-partially

Idem as Invention 1, but limited to lucPpe-2[T249M], respectively SEQ ID No. 12, where the reference beetle luciferase is lucPpe-2;

2. Claims: 47-57,75

A method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated polynucleotide

sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each comrising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the enzyme is DNA polymerase or RNA polymerase, chloramphenicol-acetyltransferase, beta-glucoronidase or beta-galactosidase; an enzyme which is encoded by the polynucleotide sequence of said method;

3. Claims: 58-partially, (59-64,69-72,74)-complete

A method to prepare an enzyme that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence, said method wherein said enzyme is DNA or RNA polymerase; a polynucleotide sequence obtained by said m

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.

Motivation of lack of unity

Thermostable beetle luciferase are well known in the prior art. EP0524448 describes a thermostable luciferase of a firefly wherein an amino acid at the 217-position is converted to a hydrophobic amino acid, which results in enhanced thermostability. Improved thermo- stability of

the North american firefly luciferase was gained by replacing the glutamate equivalent to that at position 354 of Photinus pyralis luciferase or 356 of Luciola with an alternative amino acid (WO9525798, Biochem. J 319:343,1996). D. Squirrell et all described a recombinant mutant luciferase from Photinus pyralis having an amino acid 245-mutation with improved thermostability (WO9846729). Moreover K. Wood et al. described a bundle of different thermostable luciferases derived by mutating wild type lucPpl and/or lucPpe2 from Pyrophorus plagiophthalamus and Photuris pennsylvanica (WO9914336).

-In the light of the prior art, a first problem of underlying application can be defined as the provisions of further thermostable beetle luciferases. The solutions as described and claimed could be summerized in:

a.) a second luciferase that has enhanced resistance to an inhibitor of the luciferase relative to a first reference beetle luciferase; said second luciferase which comprises a plurality of amino acid substitutions relative to the reference beetle luciferase; said second luciferase wherein the reference luciferase is LucPpe-2 (luciferase from Photuris pennsylvanica); luciferases: luc133-1B2 and luc146-1H2 which comprise SEQ ID Nos. 44 and 45; an isolated and purified nucleic acid molecule comprising a nucleic acid sequence SEQ ID Nos. 42 and 43; use of said luciferases and polynucleotides; method to prepare said luciferases that are resistant to an inhibitor; and

b.) further thermostable luciferases, derived by mutating lucPpe-2 from Photuris pennsylvanica and/or lucPpl from Pyrophorus plagiophthalamus:
-luc49-7C6 (SEQ ID Nos. 1,14);
-luc78-0B10 (SEQ ID Nos. 6,19);
-luc90-1B5 (SEQ ID Nos. 11,24);
-luc81-6G01 (SEQ ID Nos. 13,26);
-luc81-0B11 (SEQ ID Nos. 46,47) and
-lucPpe-2[T249M] (SEQ ID No. 12).
Remark: There is no direct indication or proof that these luciferases have an enhanced resistance to an inhibitor.

-The second problem is envised and concerns an alternative method to mutate and select for enzymes with enhanced enzymological properties. The solution as provided and claimed could be summerized in: a method to prepare an enzyme which is not a beetle luciferase and which has enhanced enzymological properties, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is not a luciferase and which has at least one enhanced enzymological property from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme which is not a luciferase, wherein the first isolated polynucleotide sequence is subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has at least one enhanced enzymological property relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences, wherein the selected isolated

polynucleotide sequence is subjected to oligonucleotide mediated mutagenesis with a plurality of oligonucleotides each comrising at least one codon that encodes a consensus amino acid which is not present in the first polynucleotide sequence; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme which is not a luciferase having at least one enhanced enzymological property and comprising a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the enzyme is DNA polymerase or RNA polymerase, chloramphenicol-acetyltransferase, beta-glucoronidase or beta- galactosidase; an enzyme which is encoded by the polynucleotide sequence of said method;

-The third problem of underlying application is envised and concerns an alternative method to mutate and select for enzymes resistant to inhibitors. The solution as provided and claimed could be summerized in: a method to prepare an enzyme that is resistant to an inhibitor, comprising: a) selecting one or more isolated polynucleotide sequences encoding an enzyme which is resistant to an inhibitor from a first population of polynucleotide sequences obtained from a first isolated polynucleotide sequence encoding an enzyme subjected to conditions that yield nucleotide mutations, wherein the enzyme encoded by the one or more selected isolated polynucleotide sequences has increased resistance to an inhibitor relative to the enzyme encoded by the first isolated polynucleotide sequence; b) mutating the selected isolated polynucleotide sequence to yield a second population of polynucleotide sequences; and c) repeating step a) and step b) so as to yield a further polynucleotide sequence encoding an enzyme that is resistant to an inhibitor and comprises a plurality of amino acid substitutions relative to the enzyme encoded by the first polynucleotide sequence; said method wherein the further polynucleotide sequence encodes an enzyme that has increased thermostability relative to the first polynucleotide sequence, said method wherein said enzyme is DNA or RNA polymerase; a polynucleotide sequence obtained by said method;

In the view of the fact that thermostable beetle luciferase are already well known in the prior art, moreover due to the fact that thermostable luciferases derived by mutating the wild type enzymes lucPpl and/or lucPpe2 are state of the art, furthermore due to the fact that methods to alter wild type beetle luciferases have already been described and due to the fact that methods for nucleic acid mutagenesis respectively selection of the mutated polynucleotide sequence are also state of the art, due to the essential difference of the three problems and the corresponding solutions, and due to the essential difference of the described enhanced resistance to an inhibitor of the luciferases on one side, and the origin and mutations of the 6 thermostable luciferases one the other, which are the solution of the first problem, and due to the fact that no other technical features can be distinguished which, in the light of the prior art, could be regarded as special technical features common to these solutions, the ISA is of the opinion that there is no single inventive concept underlying the plurality of claimed inventions of the present application in the sense of 13.1 PCT. Consequently there is lack of unity and different inventions, not belonging to a common

inventive concept are formulated as the different subjects on the communication persuant to Art. 17(3)(a), PCT.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 206

Continuation of Box 3.

Claims Nos.: 76 (11-partially)

In claim 11, SEQ ID Nos. 12 and 13 do not encode any amino acid sequence of claim 9 and 10.
Claim 76 refers to itself.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Annex ? orm PCT/ISA/206

COMMUNICATIO. .ELATING TO THE RESULTS OF THE PARTIAL INTERNATIONAL SEARCH

In tional Application No

PCT/US 99/30925

1. The present communication is an Annex to the invitation to pay additional fees (Form PCT/ISA/206). It shows the results of the international search established on the parts of the international application which relate to the invention first mentioned in claims Nos.:

see 'Invitation to pay additional fees'

2. This communication is not the international search report which will be established according to Article 18 and Rule 43.

3. If the applicant does not pay any additional search fees, the information appearing in this communication will be considered as the result of the international search and will be included as such in the international search report.

4. If the applicant pays additional fees, the international search report will contain both the information appearing in this communication and the results of the international search on other parts of the international application for which such fees will have been paid.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 14336 A (HALL MARY P ;PROMEGA CORP (US); WOOD KEITH V (US)) 25 March 1999 (1999-03-25) Fig.46,47 the whole document	1-6,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,68,73

X	EP 0 524 448 A (KIKKOMAN CORP) 27 January 1993 (1993-01-27) the whole document	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73

X	WO 95 25798 A (SECR DEFENCE BRIT ;LOWE CHRISTOPHER ROBIN (GB); WHITE PETER JOHN () 28 September 1995 (1995-09-28) the whole document	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WHITE ET AL: "improved thermostability of the north american firefly luciferase: saturation mutagenesis at position 354" BIOCHEMICAL JOURNAL,GB,PORTLAND PRESS, LONDON, vol. 319, no. 319, 1996, pages 343-350-350, XP002097112 ISSN: 0264-6021 the whole document	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
X	WO 98 46729 A (MURRAY JAMES AUGUSTUS HENRY ;SECR DEFENCE (GB); LOWE CHRISTOPHER R) 22 October 1998 (1998-10-22) the whole document	1-5,8, 12,14, 16,17, 19,21, 23,24, 28-37, 58,65, 66,73
A	K.V. WOOD ET AL.: "Bioluminescent click beetles revisited" J. BIOLUMNIESCENCE AND CHEMILUMINESCENCE, vol. 4, 1989, pages 31-39, XP000906944 JOHN WILEY & SONS, LTD, NEW YORK, US cited in the application the whole document	
A	WO 95 18853 A (PROMEGA CORP) 13 July 1995 (1995-07-13) the whole document	
A	K.V. WOOD ET AL.: "Complementary DNA coding click beetle luciferases can elict bioluminescence of different colors" SCIENCE, vol. 244, 12 May 1989 (1989-05-12), pages 700-702, XP002137278 AAAS,WASHINGTON,DC,US cited in the application the whole document	
A	K.V. WOOD ET AL.: "Introduction to beetle luciferases and their applications" J. BIOLUMNIESCENCE AND CHEMILUMINESCENCE, vol. 4, no. 1, July 1989 (1989-07), pages 289-301, XP000906968 JOHN WILEY & SONS, LTD, NEW YORK, US cited in the application the whole document	

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I ational Application No
PCT/US 99/30925

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DEMENTIEVA E I ET AL: "PHYSICOCHEMICAL PROPERTIES OF RECOMBINANT LUCIOLA MINGRELICA LUCIFERASE AND ITS MUTANT FORMS" BIOCHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. EASTON, PA, vol. 1, no. 61, 1 January 1996 (1996-01-01), pages 115-119-119, XP002078631 ISSN: 0006-2960 the whole document ---	
A	L. YE ET AL.: "Cloning and sequencing of a cDNA for firefly luciferase from Photuris pennsylvaniva" BIOCHIMICA BIOPHYSICA ACTA, vol. 1339, 1997, pages 39-52, XP000909154 ELSEVIER SCIENCE, AMSTERDAM, NL cited in the application the whole document ---	
A	US 5 605 793 A (STEMMER WILLEM P C) 25 February 1997 (1997-02-25) cited in the application the whole document -----	

tent Family Annex

Information on patent family members

International Application No
PCT/US 99/30925

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9914336 A	25-03-1999	AU 9492198 A	05-04-1999
EP 0524448 A	27-01-1993	DE 69229376 D	15-07-1999
		US 5229285 A	20-07-1993
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		JP 9510610 T	28-10-1997
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		JP 10500561 T	20-01-1998
		WO 9522625 A	24-08-1995
		US 5811238 A	22-09-1998
		US 5837458 A	17-11-1998